

# ULTRASTRUCTURAL LOCALIZATION OF THE HIGH MOLECULAR WEIGHT PROTEINS ASSOCIATED WITH IN VITRO-ASSEMBLED BRAIN MICROTUBULES

WILLIAM L. DENTLER, SANDRA GRANETT, and JOEL L. ROSENBAUM. From the Department of Biology, Yale University, New Haven, Connecticut 06520

Microtubules isolated from brain extracts by in vitro assembly (1, 19, 23) are composed principally of two tubulins and two high molecular weight proteins (microtubule-associated proteins [MAPs] 1 and 2) (2, 5, 7, 20). Recently, it was demonstrated that in vitro-assembled brain microtubules (neurotubules) are coated with filaments (5, 7) which are similar to the filaments attached to neurotubules *in situ* (4, 15, 21, 24, 25), and it was suggested that the filaments are composed of the high molecular weight MAPs (5, 7, 12). In this study, microtubules were assembled in the presence and absence of the MAPs, and thin sections of the microtubules were examined by electron microscopy. The results show that the filaments only occur on microtubules assembled in the presence of the MAPs and it is therefore concluded that the filaments are composed of the high molecular weight MAPs.

## MATERIALS AND METHODS

### *Preparation of Microtubule Protein*

Microtubule protein was purified from brain homogenates of 1–3-day old chicks by a modification of the method of Shelanski et al. (19). Brains were homogenized in a motor-driven glass-Teflon homogenizer at 0°C in 1 ml of 1 mM MgSO<sub>4</sub>, 2 mM ethylene glycol bis-( $\beta$ -aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 1 mM GTP, 100 mM Pipes, pH 6.9 (PM) per gram wet weight and centrifuged at 130,000 *g* for 1 h at 4°C to yield a high-speed supernate (HSS). The HSS was diluted with an equal volume of PM containing 8 M glycerol (PMG), and incubated at 37°C for 30 min to assemble microtubules. The microtubules were collected by centrifugation at 130,000 *g* for 1 h at 25°C, resuspended in PM at 0°C ( $\frac{1}{4}$ – $\frac{1}{3}$  the volume of the HSS) using a Dounce homogenizer, and incubated at 0°C for 30 min to depolymerize the microtubules. The solution was then centrifuged at 130,000 *g* for 30 min at 4°C to pellet undissociated microtubules. The supernate from this centrifugation was called S-1 and contained once-polymerized microtubule protein. The once-polymerized microtubule protein was further purified by diluting the S-1 with an equal volume of PMG and repeating the

above procedure to yield S-2, containing twice-polymerized microtubule protein. Microtubule protein (S-1) was usually stored overnight in 8 M glycerol at –20°C.

### *Column Chromatography*

A BioGel A 1.5-m column (Bio-Rad Laboratories, Richmond, Calif.), 36 cm  $\times$  1.5 cm, was equilibrated at 4°C with PM containing 0.1 mM GTP. Approximately 60 mg of microtubule protein (S-2) was applied to the column and was eluted with the equilibration buffer. Proteins were analyzed by determination of *A*<sub>280</sub> and by the method of Lowry et al. (16). When necessary, protein fractions were concentrated in collodion bags by vacuum dialysis (Schleicher & Schuell, Inc., Keene, N. H.).

For microtubule assembly, the column load and column fractions were adjusted to 2–3 mg protein per ml and a GTP concentration of 1 mM. The samples were then incubated at 37°C for 40 min to assemble microtubules.

### *Electron Microscopy*

In vitro-assembled microtubules were pelleted at 130,000 *g* for 40 min at 25°C. The pellets were fixed for 1 h in 2% glutaraldehyde in PM, postfixed, embedded, sectioned as previously described (5), and examined with a Philips EM 300. Microtubule assembly was also assayed by negative staining with 1% uranyl acetate (18).

### *Electrophoresis*

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method described by Laemmli (14). The gels were quantitatively stained for protein with the dye fast green (11) and were scanned at 650 nm with a Gilford Model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a linear transport.

## RESULTS

Microtubule protein purified by in vitro assembly procedures was shown by SDS-polyacrylamide gel electrophoresis to be composed of four major protein bands (Fig. 1): tubulins 1 and 2, with molecular weights of 56,000 and 53,000 daltons, and MAPs 1 and 2, with molecular weights of approximately 350,000 and 300,000 daltons, respectively (2, 5, 7, 20).

Purified microtubule protein was fractionated

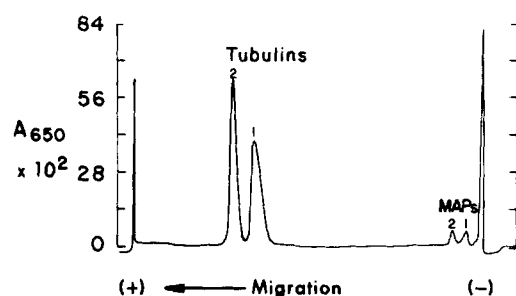


FIGURE 1 SDS-polyacrylamide gel electrophoretic pattern of unfractionated microtubule protein (S-2) (see Materials and Methods). Microtubule protein is composed principally of tubulins 1 and 2 and MAPs (microtubule-associated proteins) 1 and 2.

by molecular size with BioGel columns into two protein peaks (Fig. 2): peak 1, which eluted with the void volume, and peak 2, which was included in the column and eluted with a molecular weight of approximately 100,000 daltons. Peak 3 contained GTP.

Electrophoretic analysis (Fig. 3) showed that peak 1, like the column load, was composed of both tubulins and MAPs, although the MAPs were enriched in peak 1. The weight ratio of tubulins to MAPs was 15:1 in the column load and 2.4:1 in peak 1 (R. Sloboda, unpublished results). Peak 2 was composed of tubulins 1 and 2 and contained no detectable high molecular weight MAPs.

Negative staining at 4°C and electron microscopy showed that the column load (S-2) was composed of both 45–50-nm rings and 5–10-nm particles; peak 1 contained principally 45–50-nm rings, and peak 2 contained 5–10-nm particles. Rings were rarely observed in peak 2 fractions.

In thin sections, filaments were observed attached to microtubules assembled from peak 1 fractions (Fig. 4 *a* and *b*). In both longitudinal (Fig. 4 *a*) and cross (Fig. 4 *b*) sections these microtubules appeared similar to microtubules assembled *in vitro* from unfractionated microtubule protein (5, 7). In contrast, microtubules assembled in the absence of high molecular weight proteins from peak 2 fractions had smooth walls with no filaments attached (Fig. 4 *c* and *d*) and packed together more tightly during centrifugation.

## DISCUSSION

Ultracentrifugation studies (13, 17) have shown that microtubules assembled *in vitro* can be dissociated by cold or calcium into 6S and 30–36S

components. Results reported in this study and by others have shown that cold-dissociated microtubules can be separated by molecular size using Sephadex or BioGel columns into two fractions: (a) peak 1, containing 45–50-nm rings (6, 13) and composed of tubulins 1 and 2 and MAPs 1 and 2 (6), and (b) peak 2, containing 5–10-nm particles (tubulin dimers) and composed of tubulins 1 and 2 with no detectable MAPs (6). It is likely that the 30–36S and the 6S components observed by ultracentrifugation are analogous to the peak 1 rings and peak 2 particles, respectively.

Microtubule assembly in peak 1 fractions was rapid and was detectable by electron microscopy, viscometry, and turbidity (measured at 345 nm). In contrast, very few microtubules were assembled in peak 2 fractions and this assembly was not measurable either by viscometry or by turbidity; it could only be detected by electron microscopy. Other results, not reported here, showed that rings were not present in peak 2 fractions either before microtubule assembly or upon cold dissociation of the microtubules. Microtubule assembly in peak 2 fractions could be stimulated by the addition of small amounts of peak 1 fractions, which contained both rings and MAPs. To determine whether this stimulation of microtubule assembly by peak 1 fractions is due to the high molecular weight MAPs and/or the ring-shaped tubulin aggregates, it will be necessary to purify the MAPs. Recently, such a purification has been reported by Gaskin et al. (7).

It has been shown that *in vitro*-assembled microtubules are associated with filaments (5, 7) which are similar to the filaments attached to microtubules *in situ* (4, 15, 21, 24, 25). In the present study, these filaments were found only on microtubules which had been assembled in the presence of the high molecular weight MAPs. This shows, therefore, that the filaments are composed of the MAPs. Kirkpatrick et al. demonstrated by SDS-polyacrylamide gel electrophoresis that high molecular weight proteins are associated with *intact* microtubules isolated from brain homogenates (12). This indicates that the association of MAPs with *in vitro*-assembled microtubules is not an artifact of the assembly procedure.

It is of interest that the MAPs which form the filaments have several characteristics in common with dynein, the ATPase which forms the arms attached to flagellar outer doublet microtubules and which provides the motile force for flagellar movement (8–10, 22). On SDS-polyacrylamide

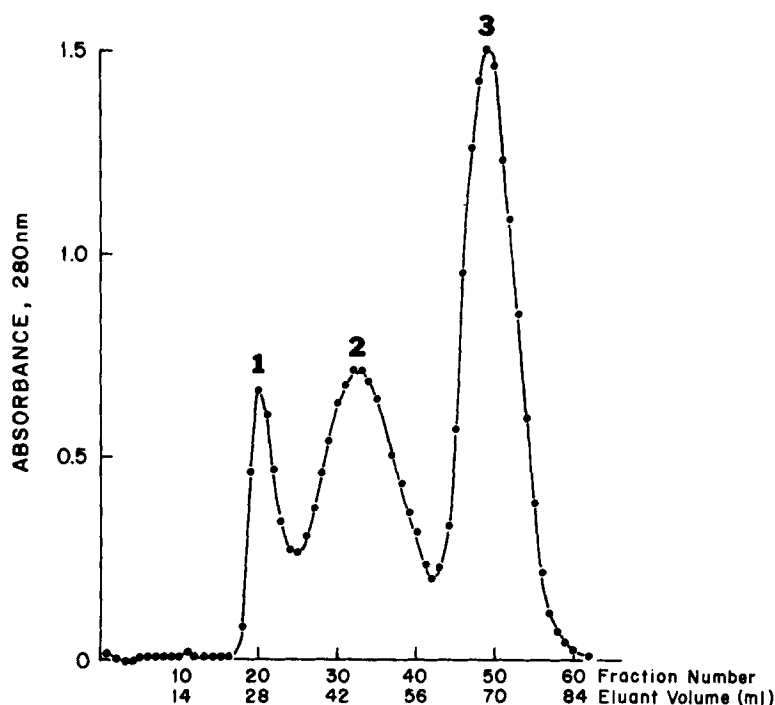


FIGURE 2 Elution profile of microtubule protein (S-2) on a BioGel A 1.5-m column. Peak 1 eluted in the void volume; peak 2 was included in the column and eluted at a molecular weight of approximately 100,000 daltons. Peak 3 was composed of GTP.

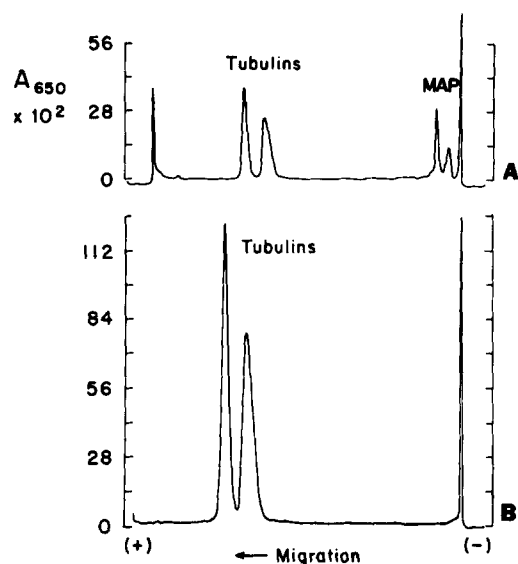


FIGURE 3 SDS-polyacrylamide gel electrophoretic analysis of microtubule protein fractionated on BioGel columns. (A) Peak 1, composed of tubulins 1 and 2 and MAPs 1 and 2. MAPs are enriched in peak 1 fractions when compared to unfractionated microtubule protein (Fig. 1). (B) Peak 2, composed of tubulins 1 and 2. No MAPs are detectable even if the gels are heavily overloaded.

gels, the mobilities of MAPs 1 and 2 are similar, but not identical, to those of flagellar dynein (Tyler and Rosenbaum, unpublished results), and MAPs may have an ATPase activity (3, 5), although this has not been clearly established (7). Furthermore, it has been reported that MAP-2 is the preferred substrate for an endogenous cyclic-AMP-stimulated protein kinase which is also associated with *in vitro*-assembled microtubules (20).

In light of studies showing the association of neurotubules with organelles in nerve axons (21), it will be important to determine if the neurotubule-associated filaments, composed of MAPs, are involved in axoplasmic transport.

#### SUMMARY

Microtubules assembled *in vitro* in the absence of high molecular weight proteins (MAPs) lack the filaments which are attached to microtubules assembled in the presence of MAPs. This indicates that the filaments attached to microtubules are composed of the high molecular weight MAPs.

This work was supported by National Institutes of Health grant GM 14642-07 and National Science Foundation grant GB 36758 to J. L. Rosenbaum. W. L.

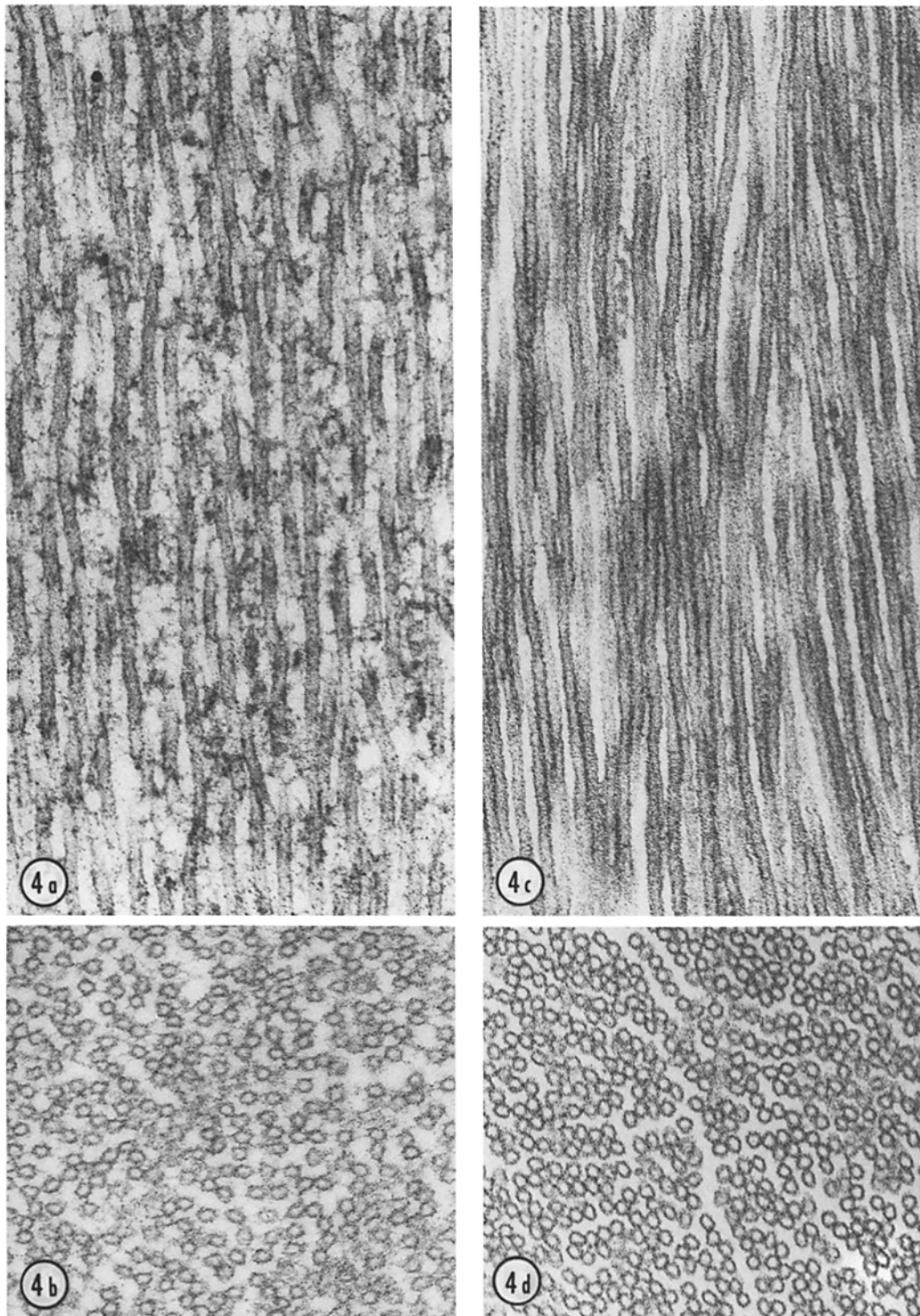


FIGURE 4 Thin sections of microtubules assembled from peak 1 fractions (*a* and *b*) and from peak 2 fractions (*c* and *d*). Filaments are only observed attached to microtubules assembled in the presence of the high molecular weight MAPs (*a* and *b*). Microtubules assembled in the absence of the MAPs (*c* and *d*) lack the filaments and pack more tightly together during centrifugation than do microtubules with filaments.  $\times 100,000$ .

Dentler is a United States Public Health Service postdoctoral fellow (GM 55-595).

Received for publication 24 October 1974, and in revised form 24 December 1974.

## REFERENCES

1. BORISY, G. G., and J. B. OLMSTED. 1972. Nucleated assembly of microtubules in porcine brain extracts. *Science (Wash. D. C.)*. **177**:1196-1197.
2. BORISY, G. G., J. B. OLMSTED, J. M. MARCUM, and C. ALLEN. 1974. Microtubule assembly in vitro. *Fed. Proc.* **33**:167-174.
3. BURNS, R. G., and T. D. POLLARD. 1974. A dynein-like protein from brain. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **40**:274-280.
4. BURTON, P. R., and H. L. FERNANDEZ. 1973. Delineation by lanthanum staining of filamentous elements associated with the surfaces of axonal microtubules. *J. Cell Sci.* **12**:567-584.
5. DENTLER, W. L., S. GRANETT, G. B. WITMAN, and J. L. ROSENBAUM. 1974. Directionality of brain microtubule assembly in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1710-1714.
6. ERICKSON, H. P. 1975. Microtubule assembly: directly from preformed, ring-shaped protofilaments. *J. Supramol. Struct.* In press.
7. GASKIN, F., S. B. KRAMER, C. R. CANTOR, R. ADELSTEIN, and M. L. SHELSKI. 1974. A dynein-like protein associated with neurotubules. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **40**:281-286.
8. GIBBONS, B. H., and I. R. GIBBONS. 1972. The effect of partial extraction of dynein arms on the movement of reactivated sea urchin sperm. *J. Cell Biol.* **54**:75-97.
9. GIBBONS, I. R. 1965. Chemical dissection of cilia. *Arch. Biol.* **76**:317-352.
10. GIBBONS, I. R., and E. FRONK. 1972. Some properties of bound and soluble dynein from sea urchin sperm flagella. *J. Cell Biol.* **54**:365-381.
11. GOROVSKY, M. A., K. CARLSON, and J. L. ROSENBAUM. 1970. Simple method for quantitative densitometry of polyacrylamide gels using fast green. *Anal. Biochem.* **35**:359-370.
12. KIRKPATRICK, J. B., L. HYAMS, V. L. THOMAS, and P. M. HOWLEY. 1970. Purification of intact microtubules from brain. *J. Cell Biol.* **47**:384-394.
13. KIRSCHNER, M. W., R. C. WILLIAMS, M. WEINGARTEN, and J. C. GERHART. 1974. Microtubules from mammalian brain: some properties of their depolymerization products and a proposed mechanism of assembly and disassembly. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1159-1163.
14. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature (Lond.)*. **227**:680-685.
15. LANE, N. J., and J. E. TREHERNE. 1970. Lanthanum staining of neurotubules in axons from cockroach ganglia. *J. Cell Sci.* **7**:217-231.
16. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
17. MARCUM, J. M., and G. G. BORISY. 1974. Sedimentation velocity analysis of intermediates in microtubule assembly. *Fed. Proc.* **33**:1231.
18. ROSENBAUM, J. L., L. I. BINDER, S. GRANETT, W. L. DENTLER, W. J. SNELL, R. SLOBODA, and L. HAIMO. 1975. Directionality and rate of assembly of chick brain tubulin onto pieces of neurotubules, flagellar axonemes, and basal bodies. *Ann. N. Y. Acad. Sci.* In press.
19. SHELSKI, M. L., F. GASKIN, and C. R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765-768.
20. SLOBODA, R. D., S. A. RUDOLPH, J. L. ROSENBAUM, and P. GREENGARD. 1975. Cyclic AMP-dependent endogenous phosphorylation of a microtubule-associated protein. *Proc. Natl. Acad. Sci. U. S. A.* In press.
21. SMITH, D. S., U. JARLFORS, and B. F. CAMERON. 1975. Morphological evidence for the participation of microtubules in axonal transport. *Ann. N. Y. Acad. Sci.* In press.
22. SUMMERS, K. E., and I. R. GIBBONS. 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea urchin sperm. *Proc. Natl. Acad. Sci. U. S. A.* **68**:3092-3096.
23. WEISENBERG, R. C. 1972. Microtubule formation in vitro in solutions containing low calcium concentration. *Science (Wash. D. C.)*. **177**:1104-1105.
24. WUERKER, R. B., and S. L. PALAY. 1969. Neurofilaments and microtubules in anterior horn cells of the rat. *Tissue Cell.* **1**:387-402.
25. YAMADA, K. M., B. S. SPOONER, and N. K. WESSELLS. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49**:614-635.